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Serological and molecular survey of tick-borne zoonotic pathogens including severe fever with thrombocytopenia syndrome virus in wild boars in Miyazaki Prefecture, Japan

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Abstract

Background: Miyazaki Prefecture is one of the hotspots of severe fever with thrombocytopenia syndrome (SFTS) cases and related deaths in Japan since 2013 and other pathogens of tick-borne diseases (TBDs). Japanese spotted fever and scrub typhus are also endemic in this region.

Objectives: A total of 105 wild boars, hunted in 2009, were serologically examined as sentinels for TBDs to indirectly demonstrate the potential hazard of ticks transmitting pathogens to humans in the studied area.

Methods: The collected blood and spleens of the wild boars underwent serological and molecular tests for SFTSV, *Rickettsia japonica* (Rj) [antibody to spotted fever group rickettsiae (SFGR) were tested by using species-common antigen], and *Orientia tsutsugamushi* (Ot).

Results: Seroprevalences of SFTSV, SFGR, and Ot were 41.9%, 29.5%, and 33.3%, respectively. SFTS viral RNA was identified in 7.6% of the sera, whereas DNA of Rj or Ot was not detected in any sample. In total, 43.8% of the boars possessed an infection history with SFTSV (viral gene and/or antibody). Of these, 23.8% had multiple-infection history with SFGR and/or Ot.

Conclusions: The high prevalence of SFTSV in wild boars might reflect the high risk of exposure to the virus in the studied areas. In addition, SFTSV infection was significantly correlated with Ot infection, and so were SFGR infection and Ot infection, indicating that these pathogens have common factors for infection or transmission. These data caution of the higher risk of SFTSV infection in areas with reported cases of other TBDs.

KEYWORDS
severe fever with thrombocytopenia syndrome, tick-borne diseases, wild boar

1 | INTRODUCTION

Severe fever with thrombocytopenia syndrome (SFTS) virus (SFTSV) is a newly identified tick-associated virus and is implicated as the causative agent of SFTS, a disease first reported in China with major clinical symptoms, including fever, thrombocytopenia, gastrointestinal symptoms, and leukocytopenia (Yu et al., 2011). The virus is currently classified as a member of the order Bunyavirales, the family *Phenuiviridae*, and the genus *Bandavirus* (International Committee on Taxonomy of Viruses, 2019). Following the first report of SFTS in China in 2009, cases started being reported in Korea, Japan, Vietnam, and Taiwan (K.-H. Kim et al., 2013; Peng et al., 2020; Takahashi et al., 2014; Tran et al., 2019), raising new public health concerns in these countries because of the lack of specific therapy for the disease, and hence, the high case fatality rate (Kobayashi et al., 2020; Zhang, Zhou, et al., 2012). Although cases of human-to-human SFTSV transmission through contact with infected patients' bodily fluid have been reported (Gai et al., 2012; W. Y. Kim et al., 2015; Liu et al., 2012), the virus is considered to be predominantly transmitted by tick bites. SFTSV genomic RNA was detected in *Haemaphysalis longicornis* and other tick species in SFTS endemic areas (Casel et al., 2021). Infectious SFTSV was isolated from *H. longicornis* in Korea (Yun et al., 2016). We also successfully isolated infectious SFTSV from *Amblyomma testudinarium*, *Haemaphysalis flava*, *Haemaphysalis formosensis*, *Haemaphysalis hystricis*, and *Haemaphysalis megaspinosa* captured in the estimated location of two SFTSV-infected patients in Japan (Sato et al., 2021). Molecular detection or isolation of SFTSV from ticks is, however, an inefficient way to understand the geographical distribution and burden of SFTSV, as the prevalence in questing ticks was extremely low, for example, 0–0.46% even in endemic areas of Japan, Korea, and China (Hayasaka et al., 2015; Park et al., 2014; Zhang, He, et al., 2012), probably because the viral copy numbers appeared to be very low in PCR-positive ticks and the places inhabited by SFTSV-harbouring ticks are quite small and difficult to identify.

On the other hand, antibodies against SFTSV were identified in various domestic animals, including goats, sheep, cattle, dogs, and pigs, and in wildlife, including rodents, deer, and boars in endemic countries and the SFTSV carriage was also discussed for cattle, cats, goats, and rodents from which SFTS viral RNA was detected (Chen et al., 2019). Although most of such infected animals were considered to be asymptomatic, SFTS clinical cases in cats (including a cheetah) (Matsuno et al., 2018; Matsuu et al., 2019) and dogs (Nam et al., 2020) and zoonotic transmission through contact with infected cats were recently reported (Kida et al., 2019; Yamanaka et al., 2020). This indicates that infected humans and companion animals were inadvertently involved in the SFTS viral ecology maintained between ticks and wildlife. The natural host of the virus is, however, still unknown.

Since 2013, more than 517 SFTS cases have been reported in western Japan as of 27 May 2020, and the endemic area has been extended to the east (National Institute of Infectious Diseases Japan, 2020). Miyazaki Prefecture, located in the southwest coastal area of Japan, is one of the hotspots for SFTS-related death, with the largest reported case number, accounting for 14% (72 of 517) of all cases in Japan. These cases were found throughout this prefecture, especially along the boundary between the mountainous area and the plain (Yasuo & Nishiura, 2019). Miyazaki Prefecture is also an endemic area for other tick-borne diseases (TBDs), including Japanese spotted fever and scrub typhus (Tsutsugamushi disease) caused by *Rickettsia japonica* (Rj) and *Orientia tsutsugamushi* (Ot), respectively (Mahara, 1997; Matsui et al., 2009). The former uses wild mice as a reservoir (Yamamoto et al., 1992) and is transmitted by *H. hystricis*, *Dermacentor taiwanensis*, and *H. flava* (Ando & Fujita, 2013; Ishikura et al., 2002). The latter uses larval trombiculid mites both as a reservoir and for transmission (chiggers, *Leptotrombidium* spp.) and is maintained solely by vertical transmission among the mites (Kawamura et al., 1995b).

Wild boars represent one of the most common wildlife species in Japan, and they are always exposed to a large number of ticks and mites in their habitat in woodland and bush areas (Merrill et al., 2018), which might influence the spatial distribution of TBDs, and possibly cause overlapping of several TBD hotspots in endemic regions. Wild boar is thus an ideal sentinel animal for epidemiological surveys of TBDs. To understand the distribution of TBDs in the natural environment in an endemic region and evaluate the potential hazard of tick-associated infections to humans, this study therefore assessed the infection status and prevalence of SFTSV, Rj, and Ot in the wild boar (*Sus scrofa*) population in Miyazaki by serological and molecular tests.

2 | MATERIALS AND METHODS

2.1 | Wild boar samples

From December 2009 to March 2010, blood and spleen samples were collected from 105 wild boars hunted in Miyazaki Prefecture, Japan, and were stored in a refrigerator (4°C) and a freezer (−20°C), respectively, until shipping. Date of sampling, geographical information, and biological data (sex, estimated age, or body weight) were collected for each animal using a standardised form. Age classification was based on animal weight and/or coat colour (Hebeisen et al., 2008) of piglets (striped, < 20 kg, 4–6 months old), juveniles (reddish, 20–40 kg, 6–12 months old), subadults (black, 41–60 kg, 12–24 months old), and adults (black or silver, large size, > 60 kg, >24 months old). Within 3 weeks after collection, the samples were shipped to the laboratory of the Miyazaki Prefectural Institute for Public Health and Environment, Miyazaki, Japan. Next, serum and blood clots were separated for each blood sample, and a 1 cm cube was cut from each spleen sample and stored at −80°C until use.

2.2 | Serological tests

Serum samples were tested for SFTSV-specific antibodies (IgG) by ELISA using supernatants of either SFTSV (HB29 strain)-infected or mock HuH-7 cells as antigens, kindly provided by Dr. Shigeru Morikawa (NIID Japan), following the protocol and interpretation described by NIID Japan. Briefly, antigens were diluted 800-fold with phosphate-buffered saline (PBS), and 100 μl was added per well in 96-well

microplates (MaxiSorp, Nunc, Roskilde, Denmark), and then the plates were placed at 4°C overnight. The wells were washed with PBS containing 0.05% Tween-20 and blocked with 20% blocking buffer (Blocking One, Nacalai Tesque, Kyoto, Japan) in distilled water, and 100 μl of sera diluted at 1:100 and 1:400 was added to duplicate wells. After an incubation at 37°C for 1 h, wells were washed and incubated with Pierce Recombinant Protein A/G, Peroxidase Conjugated (Thermo Fisher Scientific, Rockford, IL, USA) at a dilution of 20,000-fold. After washing, ABTS substrate (ABTS tablet and ABTS buffer, Roche Applied Science, Mannheim, Germany) was added to each well and incubated at 37°C for 30 min on a shaking platform, and then the absorbance was measured with a spectrophotometer (Bio-Rad, Hercules, CA) at a wavelength of 415 nm with 490 nm as a reference. Each optical density (OD) value for the wells with infected cell lysate was subtracted by that of corresponding control wells with mock cell lysate. The cutoff OD value of 0.3, set by NIID Japan, was employed after validation with domestic pig sera in this study.

Antibodies against SFGR and Ot were examined by indirect immunofluorescence assay using the YH strain of Rj (Uchida et al., 1992), and the Gilliam, Karp, Kato, Kawasaki, and Kuroki strains of Ot (Ohashi et al., 1990, 1996; Yamamoto et al., 1986), respectively, as antigens. These strains were propagated in L929 cells, and antigen preparation and indirect immunofluorescence assays were performed as described previously (Kawamura et al., 1995a). Briefly, the sera underwent twofold serial dilution from 1:40 with PBS, and were applied onto the antigens on slide glasses, followed by the addition of fluorescein-5-isothiocyanate-conjugated rabbit IgG fraction to swine IgG (whole molecule) (MP Biomedicals, LLC, Cappel Products, Irvine, CA, USA) after a 1:80 dilution with PBS with Evans Blue for contrast staining. The specific fluorescein response was observed with a fluorescein microscope (Nikon Eclipse E800; Nikon, Tokyo, Japan) equipped with a BV-2A system (excitation filter, 400–440 nm; dichroic mirror, 455 nm; barrier filter, 470 nm). Samples with specific fluorescein at 1:40 and above against the YH strain were judged as positive for SFGR-specific antibodies. Samples that tested positive for either of the Gilliam, Karp, Kato, Kawasaki, or Kuroki strain were counted as Ot positive.

2.3 | Molecular tests

To detect SFTS viral RNA, total RNA was extracted from serum samples using the QIAamp Viral RNA kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. RNA was quantified with a spectrophotometer (NanoDrop 1000; Thermo Fisher Scientific), aliquoted, and stored at −80°C until the test. Real-time reverse transcription-PCR (RT-PCR) was performed, targeting nucleoprotein (NP) and glycoprotein genes as previously described (Yoshikawa et al., 2014). A standard curve was established, based on serial dilutions of reference RNA transcribed in vitro. RT-PCR-positive samples underwent sequencing for a region (310 bp) in the NP gene (Yoshikawa et al., 2014) to generate a phylogenetic tree in which these sequences were mapped with those of the strains obtained from human SFTS cases in different areas in Japan.

Total DNA was extracted from blood clots for detection of the Rj gene and from the spleen for detection of both Rj and Ot genes by using the DNeasy Blood and Tissue kit (Qiagen). Real-time PCR was performed by using Perfect Real-time PCR (Takara Bio, Kyoto, Japan), following the manufacturer's instructions, and targeting an Rj-specific 216 bp open reading frame (Hanaoka et al., 2009) and 16S rRNA of Ot (NIID Japan, personal communication).

2.4 | Data analysis and statistics

Results are expressed as the mean value with the standard error of the mean, or as proportions with a confidence interval (CI) unless otherwise indicated. Associations between the seropositive rate and sample profiles, such as age, sex, and location, were analysed by Fisher's exact test. Associations between the antibody titre or the OD value and the sample profiles were analysed by Wilcoxon signed-rank test. Correlation among single or multiple infections with SFTSV, Ot, and SFGR was evaluated by the χ^2 test and the log-linear model. A nominal significance level of 5% ($\alpha = 0.05$) was employed for all statistical tests. All analyses were performed using the statistical program R (www.r-project.org).

3 | RESULTS

Samples were collected from 105 wild boars captured in five dispersed areas (areas A–G, Figure 3) in Miyazaki Prefecture. The animals tested in this study consisted of one juvenile, 30 subadults, and 61 adults, although age information was not available for 15 animals. There were 33 females and 47 males, although sex records were not available for 41 animals.

Of the 105 sera tested, the overall seroprevalence of TBDs as indicated by SFTSV-, SFGR-, and Ot-specific antibodies was 66.7%. The seroprevalence of SFTSV, SFGR, and Ot was 41.9% (CI, 32.3%–51.9%), 29.5% (CI, 21.0%–39.2%), and 33.3% (CI, 24.4%–43.2%), respectively (Table 1). There was no significant difference in prevalence among the three pathogens.

Eight (7.6%) of the 105 samples were positive for SFTSV genes. By contrast, Rj and Ot genes were not detected in any samples. The SFTSV copy numbers in SFTSV gene-positive samples ranged from 2.1×10^3 to 8.8×10^3 copies/ml. Of eight samples that were positive for SFTSV genes, six were also positive for SFTSV-specific antibodies (Table 2). The copy numbers were not influenced by the existence of SFTSV-specific antibody in the sera: the mean copy numbers were 5.5×10^3 copies/ml ($\pm 1.0 \times 10^3$ copies /ml) for antibody-positive samples and 5.0×10^3 copies/ml ($\pm 2.3 \times 10^3$ copies/ml) for antibody-negative samples. In the SFTSV-specific antibody-positive samples, the ELISA OD values ranged from 0.30 to 1.498, and were not influenced by the existence of SFTSV genes in the sera: mean OD values were 0.72 (±0.17) for RT-PCR-positive samples and 0.59 (±0.05) for negative samples. The phylogenetic tree with sequences obtained from four animals is shown in Figure 1. All sequences obtained from the wild boars were classified

TABLE 1 Prevalence of tick-borne disease (TBD)-associated genes and antibodies in wild boar samples collected in Miyazaki, Japan

	Antibody positive (%)	PCR positive (%)	Antibody and PCR positive (%)	Antibody and/or PCR positive (%)
SFTSV	44/105 (41.9)	8/105 (7.6)	6/105 (5.7)	46/105 (43.8)
SFGR[†]	31/105 (29.5)	0/105 (0.0)	0/105 (0.0)	31/105 (29.5)
Ot	35/105 (33.3)	0/105 (0.0)	0/105 (0.0)	35/105 (33.3)

Abbreviations: Ot, *Orientia tsutsugamushi;* SFGR, spotted fever group rickettsia; SFTSV, severe fever with thrombocytopenia syndrome virus.
[†]*Rickettsia japonica* DNA was solely targeted by PCR, while the serological test detected antibodies to SFGR.

TABLE 2 List of wild boars positive for severe fever with thrombocytopenia syndrome (SFTS) viral RNA, along with antibody test results

Sample ID	Hunting area	Age group	SFTSV ELISA (OD)	SFTS viral RNA copy number
19	E	Subadult	**0.56**	3.65
29	E	Adult	0.09	3.97
32	E	Subadult	**0.37**	3.86
36	D	Subadult	**1.50**	3.88
43	E	ND	0.10	3.77
70	E	Adult	**0.41**	3.43
85	E	Adult	**0.84**	3.33
89	D	Adult	**0.64**	3.95

Note: SFTS viral RNA copy number is expressed as $\log_{10}$ viral RNA copy number per ml of specimen. The location of each hunting area is shown in Figure 3.
Bold values represent positive ELISA results.
Abbreviations: ND, no data available; OD, optical density; SFTSV, severe fever with thrombocytopenia syndrome virus.

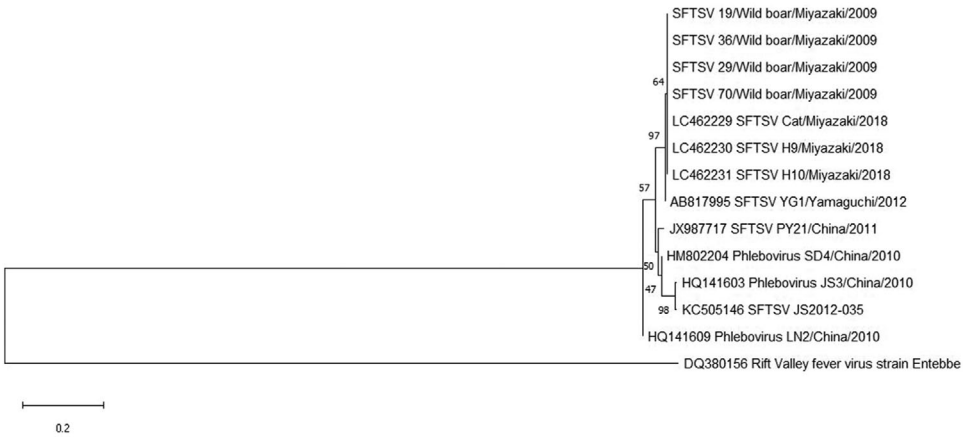

FIGURE 1 Phylogenetic analyses of severe fever with thrombocytopenia syndrome (SFTS) viral RNA based on the severe fever with thrombocytopenia syndrome virus (SFTSV) nucleoprotein (NP) gene (310 bp) obtained from serum samples from wild boars hunted in Miyazaki Prefecture, Japan, in 2009. Bold text represents the sequences obtained in this study, and the number in each name is the sample ID. Scale bars indicate nucleotide substitutions per site

as type J1 (Yoshikawa et al., 2015) and equivalently as B-2 (Yun et al., 2020), and showed homology with those from human cases reported from the prefecture.

The 46 animals with SFTSV-specific antibodies and/or SFTSV genes were counted as seropositive for SFTSV. Of these animals, 25 (54.3%) were concurrently seropositive for SFGR and/or Ot. Of these multiple seropositive samples, nine were triple positive and 16 were positive for combinations of two pathogens: five for SFTSV and SFGR, and 11 for SFTSV and Ot (Figure 2). The χ^2 test indicated a correlation between Ot infection and SFTSV infection ($p = 0.05$), and similarly for Ot infection and SFGR infection ($p = 0.002$). The best model for log expected frequency included the interaction term between Ot infection and SFTSV

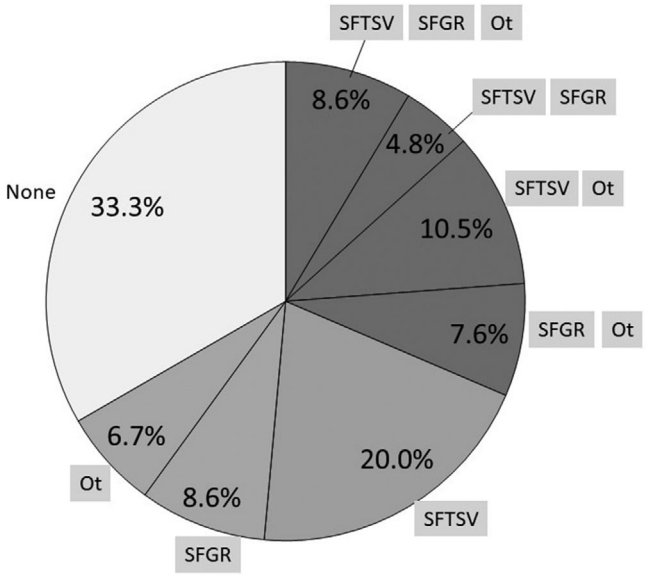

FIGURE 2 Concurrent detection of tick-borne pathogens in serum samples obtained from 105 wild boars in Miyazaki. Each section of the circle represents the proportion of the animals that tested positive for antibody to and/or nucleic acid of the pathogen(s) shown next to the section. The dark and light grey sections indicate seropositivity for multiple pathogens and a single pathogen, respectively, examined in this study. Abbreviations: Ot, *Orientia tsutsugamushi*; SFGR, spotted fever group rickettsia; SFTSV, severe fever with thrombocytopenia syndrome virus

infection and that between Ot infection and SFGR infection, in addition to the sole infections with Ot, SFTSV, and SFGR.

Sex, age, and the captured area of the wild boars in this study are shown in Table 3. None of these factors had a significant correlation with TBD prevalence, SFTS viral copy number, or the antibody titre (OD value for SFTSV) for each pathogen.

To compare the spatial distribution of SFTSV-positive wild boars and the risk of human infection, SFTSV prevalence in each area was mapped out and overlaid with geographic information system data of the suspected infection sites for SFTS patients reported from 2013 to 2017 (National Epidemiological Surveillance of Infectious Diseases (NESID) (Figure 3).

4 | DISCUSSION

In this study, the prevalence of SFTSV in wild boars captured in different areas in Miyazaki Prefecture in 2009 was greater than 40%. Although the oldest SFTS case in the prefecture was identified by a retrospective study with a stored serum sample that was collected from a patient in 2012 (Takahashi et al., 2014), it seems that the potential hazard of SFTSV infection was widely distributed in the prefecture before that time. Serological evidence of SFTSV infection in wild boars was previously demonstrated in other endemic prefectures, such as Nagasaki Prefecture (Hayasaka et al., 2016) and Yamaguchi Prefecture (Morikawa et al., 2016). On the other hand, no seropositive wild boars have been identified in non-endemic prefectures in Japan (Lundu et al., 2018; Okada et al., 2020). The geographical distribution of SFTSV-infected wild boars identified in this study was almost consistent with that of human SFTS cases (Figure 2). In addition, SFTSV genes detected from the RT-PCR-positive wild boars in this study had homology to those obtained from the sera of SFTS patients in the prefecture. Sero-surveys on wild boars as sentinel animals would therefore be a good reflection of the risk of viral infection for humans in studied areas.

Of 105 serum samples, eight were positive for SFTSV RNA, although all sampled animals were apparently healthy with no emaciation or notable gross findings in their organs. By comparison, none tested positive for Rj or Ot DNA. This indicates that wild boars might be one of the carriers of SFTSV, while playing a role as a carrier of the vectors harbouring Rj and Ot rather than as a reservoir of these rickettsia. Considering the increasing habitat range of wild boars in the country (Ministry of the Environment Japan, 2015), this animal might contribute to changes in, or even the expansion of, endemic areas of these diseases by carrying vector ticks or the virus from their original habitats to other ecosystems. As the contribution of wild sika deer (*Cervus nippon*) to changes in tick fauna (Tsukada et al., 2014; Yamauchi et al., 2009) and the strong correlation between SFTSV seroprevalence in sika deer and the number of SFTS case reports in each endemic prefecture (Morikawa et al., 2016) have been demonstrated in the country, the involvement of wild boars in TBD outbreaks may also need to be brought to attention.

On the other hand, six of eight animals possessing SFTS viral RNA also had SFTSV-specific antibodies in their blood (Table 2). Their low viral loads generally indicate fading viraemic phase after seroconversion in those animals though viral load kinetics of SFTSV in animals remain poorly characterised. The same phenomenon was observed in wild sika deer (*C. nippon*) in our preliminary study (data not shown). To the best of our knowledge, persistent infection of SFTSV in any animal has not yet been demonstrated, although infectious SFTSV was isolated from a range of wild and domestic animal species (Ni et al., 2015; Niu et al., 2013), and SFTSV RNA was detected in sheep with SFTSV-neutralising antibodies (Niu et al., 2013). As is the case for hantavirus (Fields et al., 1996), persistent SFTSV infection in wild animals may contribute to a sylvatic cycle of SFTSV.

Our results showed that 56.5% of SFTSV-infected wild boars had infection histories of SFGR and/or Ot, indicating simultaneous and/or sequential infection of several TBD pathogens in one animal. A significant correlation was observed between SFTSV infection and Ot infection and between Ot infection and SFFR infection according to the best-fitted log-linear model, indicating that there are common factors shared by several tick-borne pathogens or by their vectors in areas where these pathogens are endemic. This finding supports a report that clarified the high prevalence of SFTS among patients suspected of having scrub typhus in scrub typhus-endemic areas in South Korea (Wi et al., 2016). The previous study, in addition, found several cases indicating co-infection with Ot and SFTSV. By comparison, a correlation was not detected between SFTSV infection and SFGR infection in this study. Although the limited number of studied animals might be one of the reasons, the host preference of ticks (Fujimoto et al., 1986; Merrill

TABLE 3 Distribution of tick-borne diseases (TBDs) in wild boars, hunted in Miyazaki Prefecture, by categories of the sampled animals

Category		Prevalence and median OD value or antibody titre of seropositive samples		
		SFTSV (OD value, %)	Rj (antibody titre, %)	Ot (antibody titre, %)
Age class	Juvenile ($n = 1$)	100.0 (0.51)	0 (–)	0 (–)
	Subadults ($n = 30$)	60.0[a] (0.57)	43.3 (80)	46.7 (60)
	Adults ($n = 61$)	32.8[b] (0.38)	21.3 (80)	29.5 (60)
Sex	Male ($n = 37$)	45.9 (0.53)	27.0 (80)	27.0 (60)
	Female ($n = 29$)	37.9 (0.57)	24.1 (80)	31.0 (80)
Area	A ($n = 6$)	33.3 (0.68)	16.7 (40)	16.7 (160)
	B ($n = 2$)	0 (–)	0 (–)	0 (–)
	C ($n = 9$)	22.2 (0.57)	55.6 (80)	44.4 (60)
	D ($n = 32$)	59.4 (0.53)	28.1 (80)	50.0 (60)
	E ($n = 56$)	41.1 (0.41)	28.6 (80)	25.0 (40)
Total		43.8	29.5	33.3

Note: In the association analysis of prevalence and antibody titres, the "juvenile" age class was omitted, as there was only one individual in that class. a–b, $p = 0.02455$.

Abbreviations: Ot, *Orientia tsutsugamushi*; Rj, *Rickettsia japonica;* SFTSV, severe fever with thrombocytopenia syndrome virus.

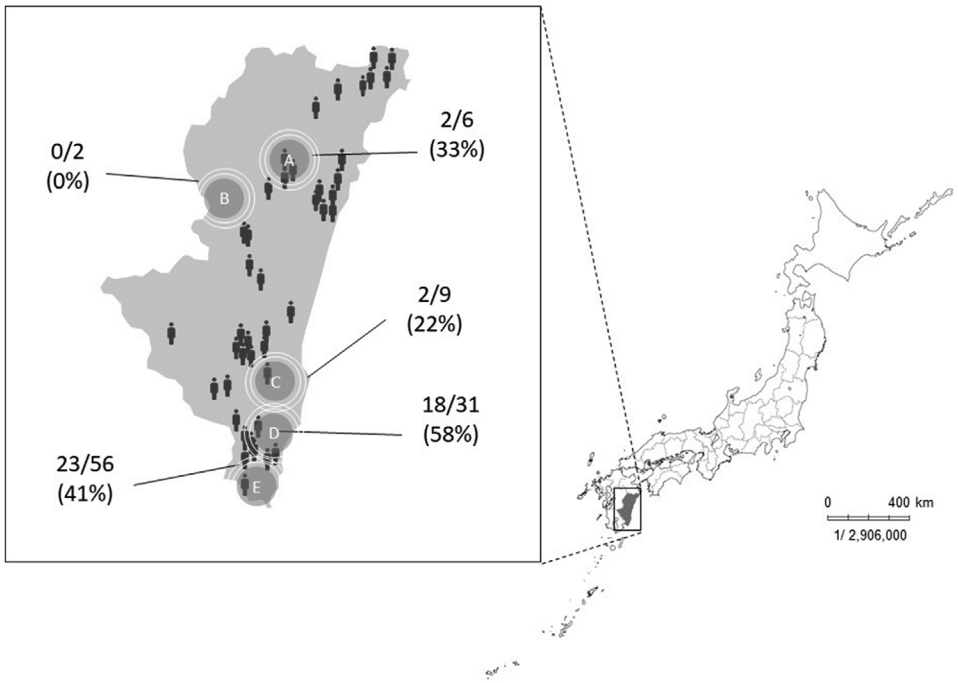

FIGURE 3 Geographic distribution of the estimated locations of human severe fever with thrombocytopenia syndrome (SFTS) infection cases reported from 2013 to 2017 and of the locations where the examined wild boars were hunted in 2009 in Miyazaki Prefecture, Japan, with severe fever with thrombocytopenia syndrome virus (SFTSV) seroprevalence in the wild boars. One human silhouette represents one human SFTS case estimated to be infected in the location. The locations where the wild boars were hunted clustered in five groups shown as A–G with a grid-based clustering technique. The number and percentage for each group show the seroprevalence of SFTSV-specific antibodies and/or viral RNA in the examined sera

et al., 2018) could affect infection opportunities of these pathogens, as well as the tick preference of the pathogens (Thu et al., 2019). A retrospective investigation in an SFTSV-endemic region in China, however, identified SFTSV-SFGR co-infected patients with more fatal outcomes (Lu et al., 2016). These findings highlight not only the importance of differential diagnosis, but also the potential necessity of raising predictive public awareness of SFTS, especially in areas where no SFTS cases have been reported but other TBDs have been reported. In addition to known risk factors associated with SFTSV infection, such as occupational and geographical risks identified in previous studies (Ding et al.,

2014; Liang et al., 2014; Yasuo & Nishiura, 2019), further studies on the distributional overlap of different TBD-causing pathogens will provide valuable insights into the ecology of SFTSV from the aspect of potential transmission opportunities.

This study has several limitations. First, 105 wild boars were included in this study, but they may not necessarily be representative of the population of interest. Second, the number of animals for each hunting area drastically varied. These limitations might affect the correlation analysis.

5 | CONCLUSION

This study demonstrates that causative agents of TBDs including SFTS were widespread in the wild boar population in Miyazaki before the identification of the prefecture's first SFTS case. Although the role of wild boars in the ecosystem of SFTSV remains unclear, our data indicate that wild boars can be sentinel animals for SFTSV epidemiology, and might be one of the carriers of SFTSV, while they play a role only as carriers of vectors for Rj and Ot rather than as a reservoir of these rickettsia. These animals might spread these diseases by carrying the vectors or the viruses from their original habitats to other ecosystems. The frequent co-infection status of different TBDs in the examined animals indicates the existence of common factors for transmission of these TBD-causing pathogens in endemic areas.

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ETHICS STATEMENT

All samples originated from dead wildlife, which were hunted or legally shot for population control by authorised hunters. Although no ethical approval or permit for this sampling was required, the hunting and euthanasia methods employed here were confirmed to follow the relevant guidelines set by the Mammal Society of Japan (The Committee of Reviewing Taxon Names and Specimen Collections, 2009).

AUTHOR CONTRIBUTIONS

Conceptualisation, data curation, funding acquisition, investigation, methodology, project administration, validation, and writing – original draft: Yumi Kirino. *Conceptualisation, data curation, investigation, methodology, resources, and writing – review & editing*: Seigo Yamamoto. *Investigation, methodology, and validation*: Taro Nomachi. *Investigation*: Mai Thi Ngan. *Formal analysis and investigation*: Yukiko Sato. *Formal analysis and investigation*: Putu Eka Sudaryatma. *Methodology and resources*: Junzo Norimine. *Formal analysis and methodology*: Yoshinori Fujii. *Conceptualisation, funding acquisition, methodology, and supervision*: Shuji Ando. *Funding acquisition, methodology, project administration, supervision, and writing – review & editing*: Tamaki Okabayashi.

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